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(54) **PHARMACEUTICAL PRODUCT  
 COMPRISING TRANSGENIC POLLEN  
 EXPRESSING HETEROLOGOUS  
 POLYPEPTIDES**

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 CPC ..... **C12N 15/8257** (2013.01); **A61K 36/00**  
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 None  
 See application file for complete search history.

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(57) **ABSTRACT**

The present invention discloses pharmaceutical products use-  
 ful for the treatment of allergies, autoimmune diseases, vac-  
 cination of mammals, as well as for in vitro diagnostics.  
 These products preferably comprise pollen grains having  
 altered protein composition by means of genetic modification  
 in the plant that produces them. The production process of the  
 present invention comprises the cultivation of genetically  
 modified plants capable of producing pollen grains having  
 modified molecular composition.

**2 Claims, 8 Drawing Sheets**

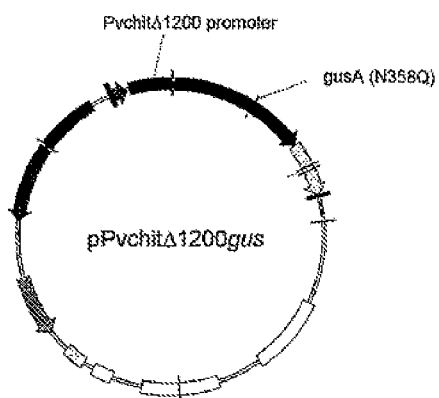


Figure 1



Figure 2

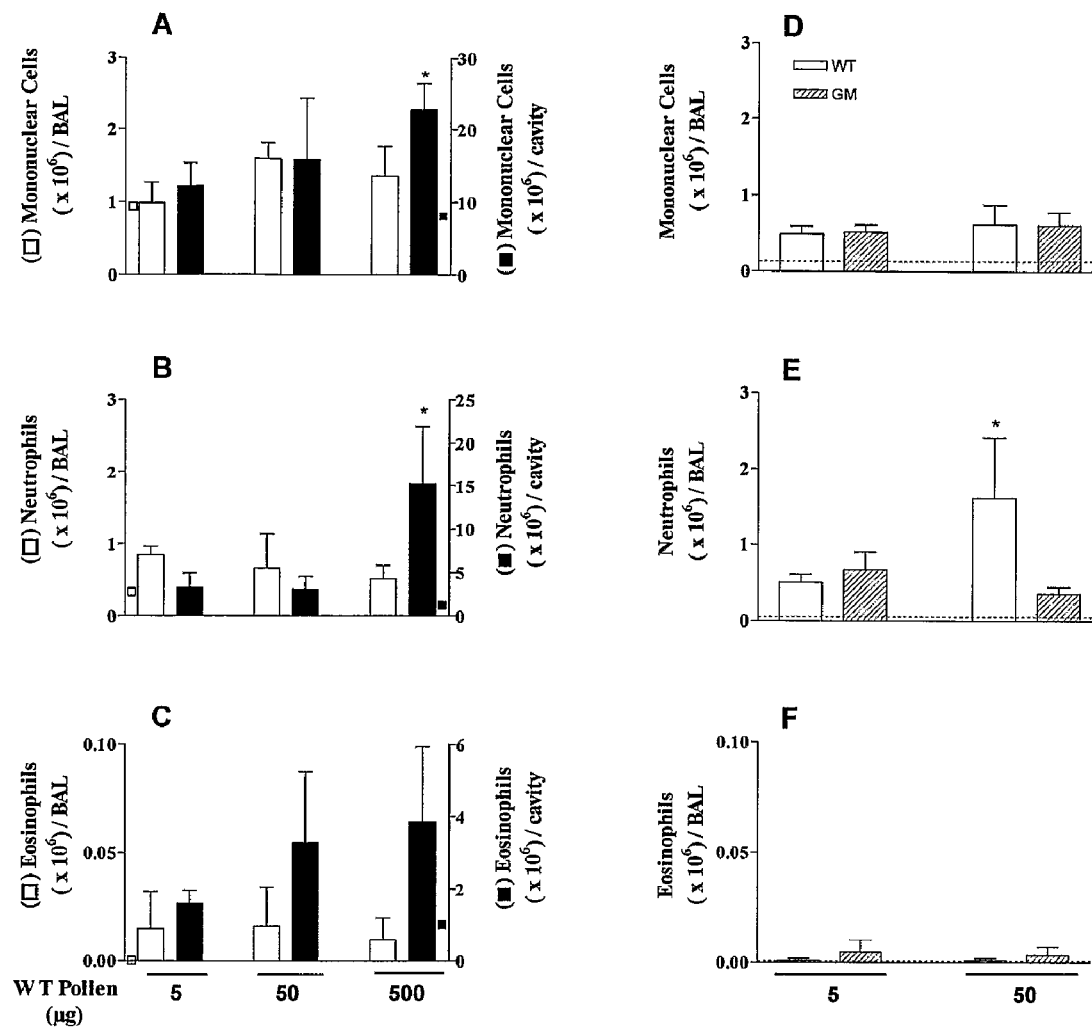


Figure 3

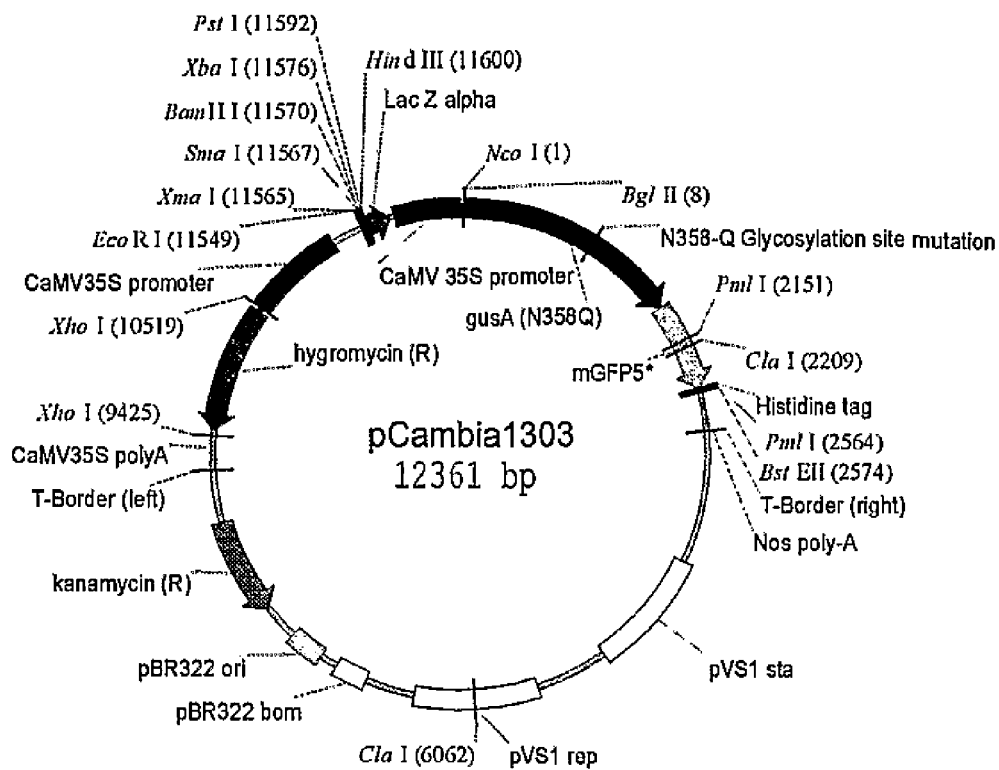


Figure 4

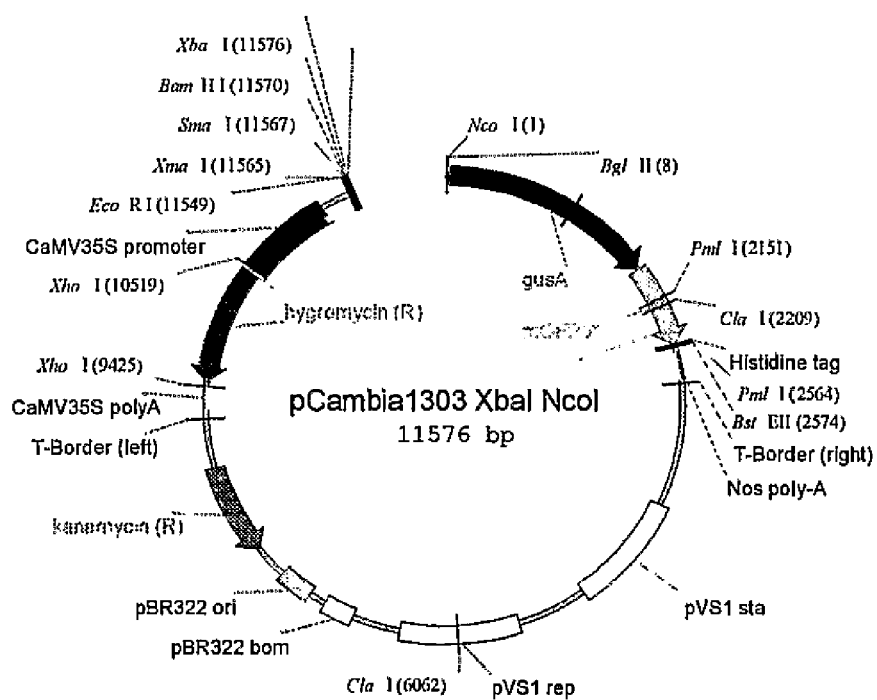


Figure 5

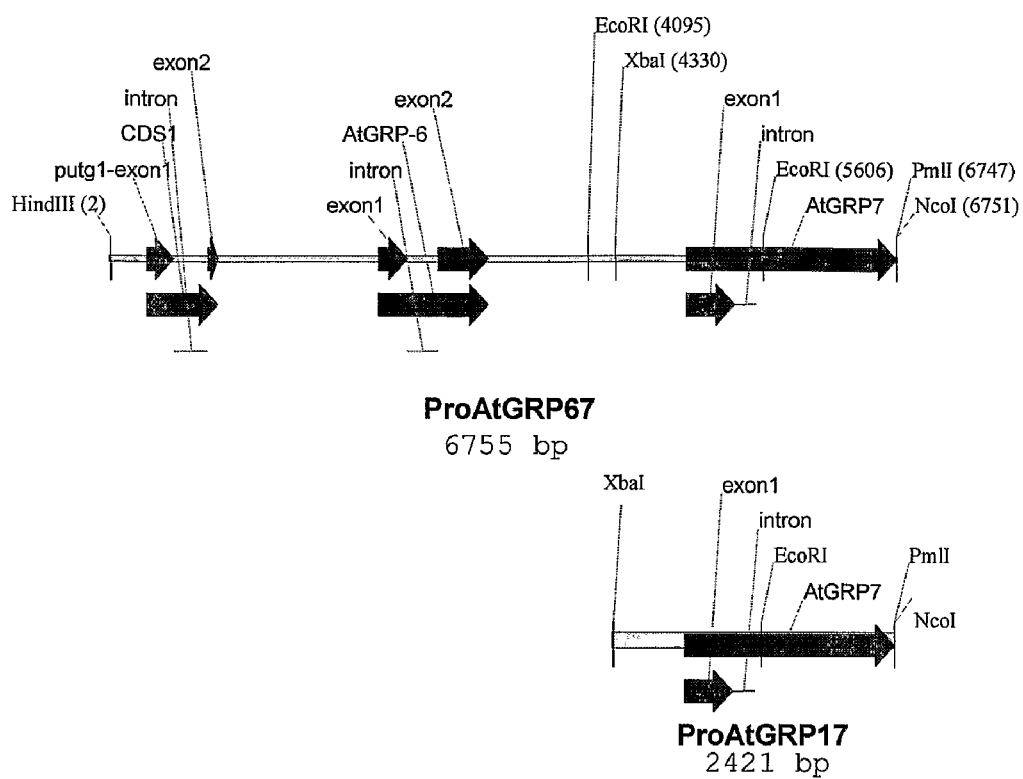


Figure 6

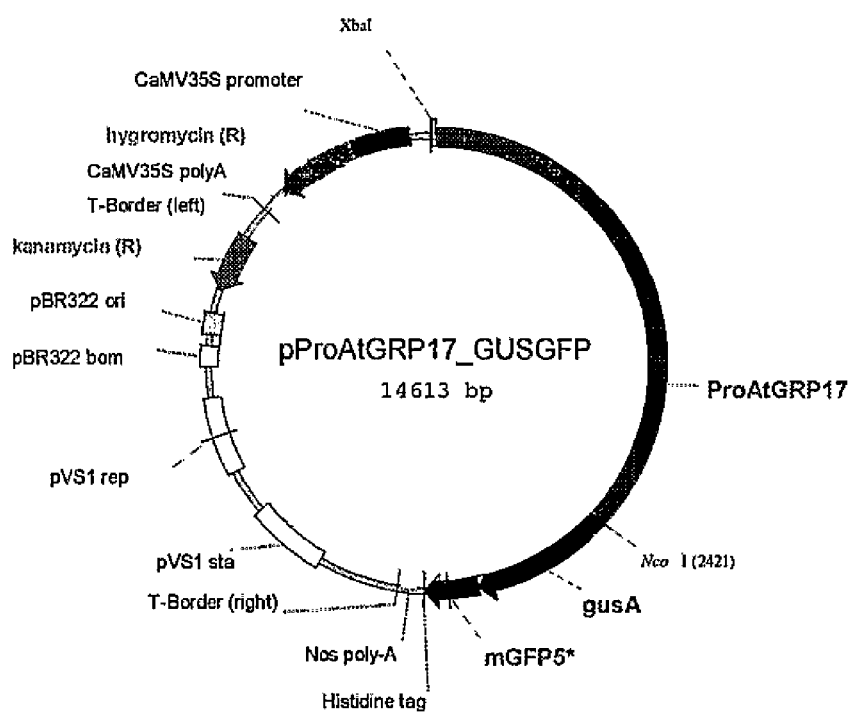


Figure 7

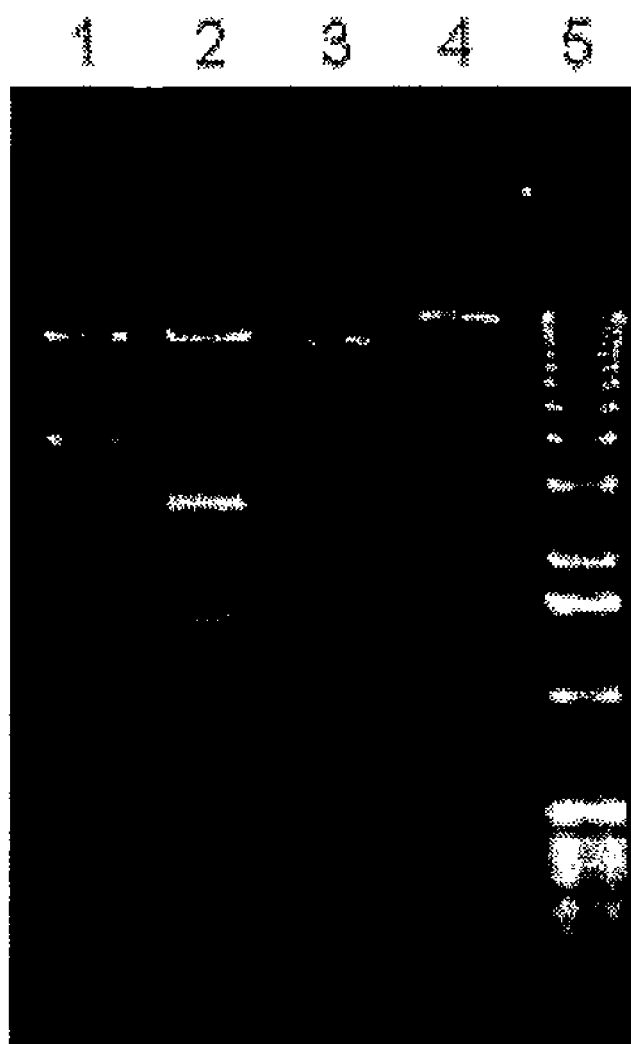


Figure 8



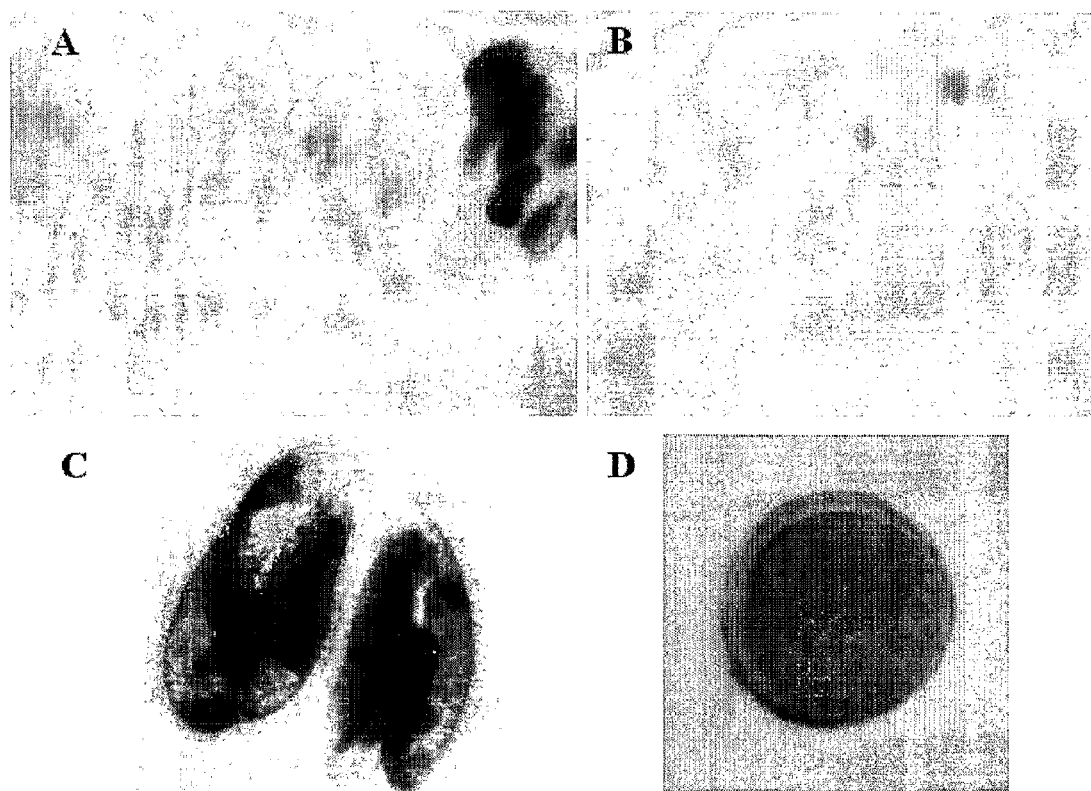


Figure 9

1

**PHARMACEUTICAL PRODUCT  
COMPRISING TRANSGENIC POLLEN  
EXPRESSING HETEROLOGOUS  
POLYPEPTIDES**

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on 30 Sep. 2013, is named 48220.001US\_S-L.txt and is 10,747 bytes in size.

FIELD OF THE INVENTION

The present invention is related to pharmaceutical products and the production processes therefor. More specifically, this invention is related to pharmaceutical products comprising pollen grains having altered protein composition through genetic modification in the plant that produces them. The production processes of the pharmaceutical products of the present invention comprise the cultivation of genetically modified plants having pollen grains with altered molecular composition, making them useful for the treatment of allergies, autoimmune diseases, as well as the vaccination of eukaryotic organisms, and for in vitro diagnosis applications.

BACKGROUND OF THE INVENTION

The processes to obtain economically relevant compounds have been changing significantly in recent years in the area of biotechnology. Until recently, the traditional industrial fermentations, for instance, were the main technological option for the production of polypeptides, enzymes, antibiotics and other substances of economic interest. Meanwhile, as the knowledge about microbiology, biochemistry and genetics of the organisms involved in the fermentative processes increased, the production technologies have also been adapted and modified. The development of molecular biology techniques was remarkable in this context: besides offering tools for the understanding of the biochemical, genetic and evolutionary mechanisms of the many species studied, molecular biology also furthered the development of countless alternatives for the industrial production of substances, as a result of the combination possibilities of the different organism's characteristics.

The present-day market shows a great demand of products and processes in the Human and/or Animal Health segment and the interest in the development of technologies in this area continues. From the industrial technology standpoint, many factors can be considered as limiting or technological "bottlenecks". Especially relevant in the production of economically important substances are the costs involved in the purification, which frequently demands a series of complex and costly steps. The determination of the cost of new therapeutic products is a very complex process, if all phases involved are to be considered—from the product conception until its placement on the market. One major factor of impact on the costs is the adopted technology, especially in the case of biological products whose chemical synthesis has not yet been developed or which presents technical or economical disadvantages. The advance of the recombinant DNA technology allowed the production of proteins and other substances in virtually unlimited quantities. On the other hand, the expression system employed has a remarkable influence on the product's nature and on the production process. A good illustrative example is the production of the Tissue Plasm-

2

nogen Activator (tPA), an anti-thrombotic agent used in the treatment of myocardium infarct, thrombosis and pulmonary embolism. This substance, when produced by *Escherichia coli*, or through the cultivation of genetically modified ovary cells from hamsters (CHO), presents significant differences regarding its structure (glycosylation, need of renaturing etc.) and production processes, which reflects on its price. In *E. coli*, 12% of the tPA's production costs correspond to the fermentative phase of the process and 88% are due to purification. On the other hand, in the production through CHO, 75% of the costs derive from the cellular cultivation and 25% from purification (Datar et al, "Process Economics of Animal Cells and Bacterial Fermentations: A Case Study Analysis of Tissue Plasminogen Activator" Bio/Technology 11:349-357, 1993). These differences are significant and can be decisively influent in the choice of the process to be used. Still other factors have a special importance in the choice of the production system, such as funds (capital) for the factory's installation, costs of raw materials, the need of qualified personnel (and its cost) as well as environmental and safety aspects.

The Vegetal Model

A safer and cheaper system to produce biologically originated substances may be idealized in transgenic plants cultivated in contained areas (greenhouses) or in agricultural areas. Furthermore, the vegetal system to produce substances of pharmaceutical interest offers various advantages, among which one might mention the absence of animal viruses and other animal cell products, as well as the absence of the typical contamination of bacterial fermentative processes, from yeasts or animal cells. In this context, the knowledge of the genetic and biochemical mechanisms of some vegetal models is reasonably wide, standing out, among other plants, *Arabidopsis thaliana*, *Nicotiana tabacum* and *Oryza sativa*. Among the main advantages of the use of the vegetal system for the production of substances with economical interest, one should emphasize the easier scale up, which is fundamental in the case of polypeptides and/or proteins of industrial interest. In the case of using microorganisms (recombinant or not), the phases involved in the scale up are normally the limiting factors of the process economy. This occurs for various reasons, among which the non-linearity of the determining relations of the process' efficiency (oxygen transfer, rheological factors, energy demand of the process etc.), the cost of the necessary production equipment and the need of adequately qualified personnel. In the case of the vegetal system, the mentioned cost components are much lower, since the scale-up tends to be simpler and linear. Besides that, in the evaluation of an industrial process' economy, the strategies seeking to eliminate the largest possible number of purification phases should be considered. Furthermore, plants are metabolically able to perform complex post-translational modifications, such as glycosylations, which widens the scope of possible peptides, antigens or vaccine candidates to be produced by plants. In this sense, the strategy of the present invention comprises the development of expression systems which take into account the most appropriate cellular and sub-cellular localizations for the desired products and, foremost, the elimination of purification steps.

The reproductive systems of *A. thaliana* and *N. tabacum* have been intensely studied in the recent years and are suitable to the logic of eliminating purification steps of the present invention. The starting point for the idealization of the present invention was the existing knowledge about the genes involved in the vegetal reproductive development and, more precisely, those related to the development of the tapetum which, for some years now, is the study object of the Laboratory for Plant Molecular Genetics of the Federal University

of Rio de Janeiro, Brazil. Former studies in that laboratory in this area comprise the state of the art of the present invention. On one hand, the knowledge about the expression of the genes involved in the formation of *A. thaliana*'s inflorescence (Franco, MSc. Dissertation, UFRJ, 1992) was helpful to understand the function of the oleosin-type proteins, more recently studied with molecular biology techniques associated to microscopy (Ferreira, PhD Thesis, UFRJ, 1997). On the other hand, there also exists knowledge about the regulation mechanisms of the codifying genes of said oleosin-type proteins, studied with the help of the .beta.-glucuronidase (GUS) gene marker (Scholte, PhD Thesis, UFRJ, 1998). Even more recently, a strategy of modifying the protein composition of the external surface of pollen grains was described by Foster et al "Modifying the pollen coat protein composition in *Brassica*", Plant Journal 31(4): 477-486, 2002, also described in the document WO 99/49063, by the same authors. However, neither these, nor any other reference known by the author make any allusion or suggestion regarding the use of genetically modified plants for the production of pharmaceutical products in tissues and/or cells of the male vegetal reproductive system, neither do they mention the use of whole, intact, pollen grains derived from genetically modified plants as pharmaceutical products to be used in immunoreactions, as vaccines or as reactive agents for diagnostics, which collectively comprise the objects of the present invention.

For the purposes of the present invention, one should understand as "tissues and cells of the male vegetal reproductive system" the tissues or cells of the male vegetal reproductive system, including the anthers, tapetum, pollen—grains, parts and combinations thereof. For the purposes of the present invention, one should also understand as "immunoreactions" all reactions that involve cells and/or molecules of the immune system of eukaryotes, including vertebrates, invertebrates, mammals and the like, including mononuclear cells such as macrophages and lymphocytes B and T, neutrophils, eosinophils, besides antigens, antibodies, cytokines and other chemical mediators of the immune system, including parts of the same and its combinations. Furthermore, for the purposes of the present invention, one should understand as "heterologous polypeptide" any amino acid sequence which is not naturally produced by the plant, but whose synthesis in it derived from the genetic modification undertaken in the plant through the present invention. Without limiting the scope of the present invention, one should emphasize as being of special importance the use of pollen grains containing at least one heterologous polypeptide.

The present invention offers means to avoid some difficulties in both the production and use of polypeptides with therapeutic and/or diagnostics interest. On one hand, pollen grains are structurally stable, probably as a consequence of the need of reproductive success. Therefore, their "evolution" in order to resist the most diverse environmental stresses is useful to the logic of using them as a product, since their high stability is favorable and desirable. In an additional aspect, whole pollen grains of the present invention may be used in certain applications, bypassing the need of purification. As examples, herein used to illustrate the present invention but not to limit its scope, whole pollen grains containing heterologous polypeptides would permit: the direct use as antigens in the production of diagnostic kits, especially for screening, and its use as a vaccine preferably delivered onto mucous membranes or injected subcutaneously.

Pollen is often associated to allergy because some people develop symptoms such as sneezing, itching, cough, nasal irritation, eye watering and asthma when exposed to the pollen of certain plants. These particles are carried in large

amounts by the air, normally during springtime. When they get in contact with the nasal mucous membranes or the throat, they may trigger allergic reactions known as polinosis or seasonal allergic rhinitis (for more details, consult Balda et al, "Tree-pollen Allergy is Efficiently Treated by Short-term Immunotherapy (STI) with Seven Preseasonal Injections of Molecular Standardized Allergens". *Allergy* 53, 740-748, 1998). As in any allergic process, the polinosis is a high sensibility to certain substances present in the pollen, and considerably varies from person to person, even though there seems to be a familiar correlation. In the majority of allergic reactions, the immune system responds to a "false alarm", mobilizing the attack against the allergen. The organism produces large quantities of specific IgEs, which bind themselves to the mastocytes in the tissues and to the basophils in the blood. When the allergen meets the IgE the liberation of histamine, prostaglandin, leucothriens and other substances occurs, thus causing the allergy symptoms (for more details, see Batanero et al, "IgE-binding and Histamine-release Capabilities of the Main Carbohydrate Component Isolated from the Major Allergen of Olive Tree Pollen, Ole e 1". *J Allergy Clin Immunol* 103, 147-153, 1999). Some strategies have been developed in order to obtain vaccines against autoimmune diseases and allergies, being generally based on the induction of tolerance. For example, known pollen grain allergens, when prepared in an encapsulated form, have shown to be efficient in the induction of tolerance by means of nasal administration. Nevertheless, the available encapsulating methods of allergens/antigens are laborious and costly, besides generally bringing about the denaturation of said allergens/antigens. The present invention's approach makes it possible to overcome these problems through the production of heterologous allergens/antigens in pollen grains.

Due to its natural ability to stimulate the production of specific IgEs and IgGs, we started from the hypothesis that the pollen could become a good candidate as a carrier and deliverer of vaccine antigens in mucous membranes, such as its direct use via nasal immunizations. Besides the already mentioned elevated structural stability of the pollen, an additional characteristic of the present invention under consideration is to enable the use of whole pollen grains as vaccines. Interestingly, one of the goals clearly expressed by the World Health Organization (WHO) is the development of new systems for the delivery of antigen vaccines to the respiratory tract. Furthermore, recent studies have demonstrated that vaccines derived from transgenic plants, when applied in the form of dry powder, seem to be the preferred solution for the lack of homogeneity in the concentrations of antigens produced in plants (for further references, see Sala et al., "Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives". *Vaccine* 21:803-808, 2003; Mielcarek et al. "Nasal vaccination using live bacterial vectors". *Advanced Drug Delivery Reviews* 51:55-69, 2001). On the other hand, the WHO's recommendation for the vaccine against tetanus (or lockjaw), for instance, is the administration of three consecutive doses of the respective antigen. This repeated administration has financial and logistical disadvantages, since some patients do not return for the second dose and because the vaccination campaigns have as a limiting factor: the need of a cold chain, considering that practically all vaccines are thermo labile. The availability of vaccines in pollen grains presents itself as capable of solving these mentioned problems, besides being easily delivered (without injections), easily standardized and endowed with an elevated thermal stability, being able to help avoid the logistics problems which are so far inherent to vaccination campaigns.

These advantages open up good perspectives for the study and development of this way of immunization in combination with the presentation form of the present invention.

Several efforts have been made in the last two decades in the sense of trying to develop subunit vaccines for human and veterinary use. The subunit vaccines are based on individual components derived from the infectious agent and, normally, have a low immunogenicity due to the absence of other cellular constituents from which they are often purified. Therefore, when developing vaccines it is desirable to plan the utilization of other substances which have the potential to increase the immune response to the antigens in question, what is normally done with the use of adjuvants. Entire cells or parts thereof can work as self-adjuvants, which is favorable for the present invention's approach. The identification of an appropriate antigen is only the first step in the development process to obtain a subunit vaccine, since adequate adjuvant systems and delivery systems of the respective antigen are also necessary. An adjuvant can be any material which increases the immune-humoral and/or cellular response to the antigen(s); it is generally accepted in literature that certain adjuvants act through the gradual liberation of the antigens to the cells of the immune system. Recent studies (Wiedermann, et al. "Modulation of an allergic immune response via the mucosal route in a murine model of inhalative type-I allergy" *Intl. Arch. Allergy Immunol.* 118:129-132, 1999) have shown that antigenic preparations in powdered form can also increase the incorporation of antigens by the antigen processing cells of the immune system. In this sense, the use of whole pollen grains, due to its powdered nature, offers this additional advantage in the case of application in vaccines. Independently of exactly knowing the specific mechanism involved, it is known that not only the cellular, but also the humoral immunity might be stimulated in various degrees, depending on the antigen, the adjuvant, the administration protocol and the species involved.

In order to develop an effective and commercially feasible vaccine, the relation between the production cost and the large-scale production capacity of the antigenic preparation and the adjuvant system should be taken into account. On the other hand, the growing number of vaccines under developed and the number of required injections for a wide-ranging immunization program for children, for instance, generates elevated costs and the preoccupation about the discontinuity potential of current vaccination programs. This makes highly desirable the availability of alternative immunization means, especially those allowing the so-called multivalent vaccination, that is, the one that permits to present multiple antigens or epitopes simultaneously. In this context, transgenic plants have been the object of several attempts to obtain "edible" vaccines, due to their notorious advantages in terms of production costs and administration. However, vaccines produced in transgenic plants destined for oral consumption have the disadvantage of passing by the gastrointestinal barrier, which destroys a significant portion of the vaccine antigens. For that reason, the state of the art shows that the amount of antigens required for an effective immune response derived from an edible transgenic plant vaccine is from 100 to 1,000 times higher than the amount of antigens necessary for an effective parenteral immunization (for further reference, see Carter III, J. E., and Langridge, W. H. R. "Plant-based vaccines for protection against infectious and autoimmune diseases". *Critical Reviews in Plant Sciences* 21(2), 93-109, 2002; Streatfield and Howard "Plant-based vaccines". *International Journal for Parasitology* 33, 479-493, 2003). Therefore, the need to develop new alternatives to overcome these difficulties persists.

The entry route of most pathogenic agents is the mucous surfaces and a large part of the infections is located in the mucous and sub mucous tissues. However, conventional injectable vaccines are poorly efficient for the induction of an immune response in mucous membranes. As a consequence, several experiments are being carried out in order to offer alternatives for the immunization in mucous membranes, which includes, for instance, the incorporation of antigens in larger particles (such as liposomes, immune stimulatory complexes, microspheres) for increasing the efficiency of the immune response in mucous tissues. On the other hand, the use of adequate adjuvants is normally very important for the stimulation of the desired type of immune response. Among the known adjuvants, and in the scope of the present invention, one might point out the vegetable oils. A primary advantage of the use of vegetable oil adjuvants over the use of mineral oils is that vegetable oils can be easier metabolized and are, therefore, more tolerable. Patent literature is rich in examples of the use of vegetable oils as adjuvants in vaccines, including, for instance, documents WO 01/95934 "The use of plant oil-bodies in vaccine delivery systems" and WO 02/00169 "Production of vaccines using transgenic plants or modified plant viruses as expression vectors and transencapsidated viral coat proteins as epitope presentation systems". Most of these patents disclose formulations and the use of water-in-oil or oil-in-water emulsions which are prepared through the mixture of pure chemical compounds. However, none of the mentioned documents reveals or suggests the use of tissues or cells from the male vegetal reproductive system, such as whole pollen grains, or even parts thereof, as an useful pharmaceutical product in immune reactions or as an antigen-adjuvant combination for immunization, preferably applicable in mucous membranes. In this context, the present invention offers a technical and commercially feasible production alternative of a pharmaceutical product which is potentially useful in the treatment of dysfunctions of the immune system of eukaryotes and which allows, among other characteristics, the production of multiple substances in one single system.

## SUMMARY OF THE INVENTION

Having in mind the prior art limitations and all the above-mentioned reports, the present invention provides pharmaceutical products as well as their production process in genetically modified plants. The products of the present invention comprise tissues and/or cells of the male vegetal reproductive system, including bioactive substances to be used in Human and/or Animal Health.

In one aspect, the present invention provides a production process of pharmaceutical products which overcome difficulties in the production, distribution, storage and logistics of said products. In another aspect, the present invention offers a production process of pharmaceutical products to be used as immune reactions, through the expression, in male vegetal reproductive tissues, of marking genes, codifying genes of antigens and/or therapeutic polypeptides. In yet another aspect, the pharmaceutical products of the present invention are useful in the immune modulation of eukaryotes, including mammals and other vertebrates as well as invertebrates such as insects and the like. From an additional point of view, the pharmaceutical products of the present invention are potentially useful in immune diagnostic reactions. These and other aspects constitute the objects of the present invention and will

be clearer through their detailed description and the corresponding attached figures, as well as the appended claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of the binary plasmid pPvchitΔ1200gus, used as vector for the reporter gene GUS under the control of part (−1200) of the bean chitinase promoter.

FIG. 2 is a photograph of a tobacco flower, clearly showing the anthers.

FIG. 3 are counts of mononuclear cells A), neutrophils B), and eosinophils C), in alveolar lavage (BAL) of rats subjected to three consecutive instillations of pollen grains in the indicated concentrations and in pleural lavage of rats subjected to intrapleural injections of pollen grains in the same concentrations. □ and ■ represent, respectively, the average values of BAL and pleural controls. C), D), and F) represent, respectively, the counts of mononuclear cells, neutrophils and eosinophils of broncho-alveolar lavage of rats subjected to three consecutive instillations of WT or GM pollen grains in the indicated concentrations. Dashed lines represent the average values of the controls instilled with saline solution. Columns represent averages±SE. P. M. and asterisks indicate statistically significant differences ( $p<0.05$ ).

FIG. 4 is a schematic representation of binary plasmid pCambia 1303, used as vector having the reporter genes GFP, GUS and of the selection markers for Kanamycin and hygromycin. Single restriction sites XbaI and NcoI and other single and double restriction sites are also indicated.

FIG. 5 is a schematic representation of the binary plasmid pCambia 1303 without the CaMV 35S promoter region, as per the cleavage with XbaI and NcoI enzymes.

FIG. 6 is a schematic representation of the ProAtGRP67 region obtained by PCR and the fragment ProAtGRP17 generated by cleavage with XbaI and NcoI enzymes. In the latter the shorter arrow indicates the relative position of exon 1 and the intron.

FIG. 7 is a schematic representation of the plasmid pProAtGRP17\_GUSGFP, resulting from the cloning of the PCR product ProAtGRP67 cleaved with XbaI and NcoI in the plasmid pCambia 1303 cleaved with the same enzymes. The 616 bp region corresponding to the part of the AtGRP17 promoter region and the AtGRP17 gene ORF are indicated as ProArGRP17. Reporter genes GUS and GFP are also indicated.

FIG. 8 shows 1% agarose gel containing the expected DNA fragments of the plasmids extracted from transformed *E. coli* XL1, obtained after cleavage with the indicated enzymes. 1, pCambiaProAtGRP17 PvuII; 2, pCambiaProAtGRP17 BglII; 3, pCambia PvuII; 4, pCambia BglII; 5, 1 kb ladder marker.

FIG. 9 shows floral structures of *A. thaliana* transformed with the plasmid pCambiaProAtGRP17GUSGFP. Panel A) shows the presence and activity of GUS on the late anthers' development, but not in the initial stages of development. Panel B) shows inflorescences of the same plant in which the activity of GUS can be seen in the anthers of immature flowers (left) and in the anthers and petals of the mature flower (right). Panel C) shows intense GUS activity on the tapetum and on the pollen grains. Panel D) shows a pollen grain with positive stain for GUS. All photographs were taken under an optical microscope.

#### DETAILED DESCRIPTION OF THE INVENTION

In order to evaluate the applicability of the use of tissues and cells from the male vegetative reproductive system as phar-

maceutical products, we evaluated situations which could be considered to be most critical to said use. Therefore, the tested applications, even though not limiting the scope of the present invention, are herein presented as the use of whole pollen grains as vaccines, immunotherapeutic agents and/or diagnostics reagents. In order to achieve this goal, genetic constructions were developed aiming to allow the expression of a reporter gene in such a way that its presence in plant subcellular structures and in pollen grains could be more easily analyzed. Different genetic constructions containing the reporter gene GUS (codifier of the β-glucuronidase enzyme) were evaluated: (i) the binary vector pDE1001 for the transformation of *N. tabacum* plants containing the reporter gene GUS under the control of the promoter PvChit (promoter of the bean chitinase, *Phaseolus vulgaris*, the genetic construction of which was detailed in Lima et al., (2002) Bean class IV chitinase promoter is modulated during plant development and under abiotic stress. *Physiologia Plantarum* 116:512-521, in a construction herein called pPvchitΔ1200gus, FIG. 1), which allows the intracellular expression of the glucuronidase (GUS) enzyme in tobacco pollen grains; and (ii) the binary vector for the transformation of plants pCambia 1303 (FIGS. 4 and 6) as the receptor of the in-frame fusion SEQ ID NO. 1-GUSGFP under the control of the promoter SEQ ID NO. 2 (formerly called AtGRP7 by de Oliveira et al. "Inflorescence-specific genes from *Arabidopsis thaliana* encoding glycine-rich proteins". *Plant J.* 3:495-507, 1993). This last construction aims to permit the expression of the fusion SEQ ID NO. 3-GUSGFP on the surface of pollen grains, in order to allow the testing of the pollen grains hereby obtained for the applications of the present invention.

#### EXAMPLE 1

##### Obtaining "WT" and "GM" Tobacco Pollen Grains

Wild tobacco plants (*N. tabacum*) from the SR1 lineage, referred to as "WT" in the present invention, and also tobacco plants transformed with the vector pPvchitΔ1200gus (FIG. 1), available from a previous study (Viviane Moreira, PhD Thesis, UFRJ, 2002, also described in Lima et al., "Bean class IV chitinase promoter is modulated during plant development and under abiotic stress". *Physiologia Plantarum* 116:512-521, 2002) were cultivated in a greenhouse until the blossoming of mature flowers. The genetically modified lineage of tobacco in the present invention is referred to as "GM", and produces pollen grains with an altered composition, containing the GUS enzyme in its interior. The following procedure was adopted in order to collect the pollen grains: as soon as the dehiscence occurred, the entire flowers (FIG. 2) were removed from the WT and GM plants and the intact anthers were transferred into Eppendorf tubes with the help of a scalpel. The average weight of the pollen grains present in each inflorescence was weighted with an analytical scale. Taking into account the difference between the total weight with anthers and the total weight without anthers (after homogenization and the subsequent removal of the pollen grains present in said anthers), the average weight of pollen grains present in each flower was equal to 1 milligram—consequently, the equivalent of 0.2 milligram for each anther, since one *N. tabacum* flower has 5 (five) anthers.

#### EXAMPLE 2

##### Evaluation of GUS Stability in "GM" Pollen Grains

The stability of pollen grains maintained at room temperature (about 25 degrees Celsius) was evaluated qualitatively through the verification of the activity of the p glucuronidase

(GUS) enzyme in the GM pollen grains. In order to do so, colorimetric reactions were performed using 1  $\mu$ L of the compound X Gluc in 100  $\mu$ L of phosphate buffer 0.1 M pH 7 and whole transgenic pollen grains containing the GUS enzyme. In the tests performed in laboratory, the activity of the GUS enzyme was kept in stock for at least one year at room temperature. In this context, it is worthwhile to emphasize that the physico-chemical conditions necessary for the maintenance of an enzyme's activity are much more restricted than the conditions necessary to maintain the immunogenicity of a polypeptide sequence. Therefore, these results suggest an elevated stability of heterologous peptides expressed in the pollen grains of *N. tabacum* "GM", which is desirable within the scope of applications of the present invention.

#### Analysis of the Immune Response of Rats to Tobacco Pollen Grains

Since the present invention relates to new production technologies and formulations of pharmaceutical products especially applicable in immune reactions, such as immune modulators, vaccines and diagnosis reagents, the immunoreactivity of pollen grains under different conditions is the starting point for the evaluation of its applicability. The approach used herein includes the study of the immune response of female Wistar rats (*Ratus norvegicus*), submitted to different ways of pollen grain exposures.

The potential of pollen grains to induce inflammation was evaluated by analyzing different administration schedules of WT tobacco pollen grains in rats. The cellular responses of animals submitted to different exposure/sensitizing schemes were monitored by counting the amount of cells present in broncho-alveolar lavage or in pleural fluid and checking the appearance of paw edemas, while the molecular responses were monitored through the observation of protein extravasation, measurement of the total serological IgE and IgG concentrations through ELISA, and of specific serological IgGs against pollen grain proteins by Western Blot.

Groups of 3 to 6 animals, as indicated, were submitted to the administration of different regimes of saline solution suspensions containing WT or GM tobacco pollen grains, in the concentrations of 0.5, 50 and 500  $\mu$ g per animal (concentrations based on the average weight of 1 mg of pollen per tobacco flower), with or without the joint administration of aluminum hydroxide (5 mg per animal) as adjuvant. The verification of the occurrence or not of any inflammation in the broncho-alveolar cavity or the pleural cavity was done respectively through broncho-alveolar or pleural washing, followed by the total leucocytes count in a Neubauer chamber and a relative count of monocytes, neutrophils and eosinophils after centrifugation on a glass slide. Both counts were carried out with the help of an optical microscope. As will become shown in the following experiments, none of the tested conditions caused a significant alteration of cell counts consistent with an inflammatory process. Additionally, none of the tested conditions produced significant alteration of the protein exudates, which would be typical of an inflammatory process. Serum samples of the animals submitted to the different experimental conditions were collected and stored for later molecular analysis. And again, none of the pollen grain administration conditions tested showed a significant increase of the total serological IgE and IgG concentrations, according to quantitative measurements through ELISA. The results of the following tables show that none of the different pollen grain administration conditions caused detectable allergic/inflammatory type reactions in animals.

#### EXAMPLE 3

#### Immune Response of Rats Submitted to a Single Intrapleural Injection

TABLE 1

Experimental conditions of pollen grain exposure to <i>R. norvegicus</i> Wistar rats and their respective results. The animals were submitted to an intrapleural injection of 5 $\mu$ g of wild tobacco pollen grains (100 $\mu$ L in saline solution). The chart shows the cell counts in pleural lavage 4 hours after the procedure.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	17	85	15	0	4.34	0.77	0
2	25	83	17	0	6.23	1.28	0
3	16	85	15	0	4.08	0.72	0
Average					4.88	0.92	0
Deviation					1.17	0.31	0
E.P.M					0.83	0.22	0

Subtitle abbreviations:

CAM Leu tot (total leucocytes counted in a Neubauer chamber);

Mono % (% of monocytes);

Eos % (% of eosinophils);

Neut % (% of neutrophils);

E.P.M. (mean standard error).

TABLE 2

Experimental conditions of pollen grain exposure to <i>R. norvegicus</i> Wistar rats and their respective results. The animals were submitted to an intrapleural injection of 50 $\mu$ g of wild tobacco pollen grains (100 $\mu$ L in saline solution). The chart shows the cell counts in pleural lavage 4 hours after the procedure.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	17	86	14	0	4.39	0.71	0
2	12	78	22	0	2.81	0.79	0
3	17	88	11	1	4.49	0.56	0.05
Average					3.89	0.69	0.02
Deviation					0.94	0.12	0.03
E.P.M					0.67	0.08	0.02

TABLE 3

Experimental conditions of pollen grain exposure to <i>R. norvegicus</i> Wistar rats and their respective results. The animals were submitted to an intrapleural injection of 500 $\mu$ g of wild tobacco pollen grains (100 $\mu$ L in saline solution). The chart shows the cell counts in pleural lavage 4 hours after the procedure.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	20	82	18	0	4.92	1.08	0
2	21	81	19	0	5.10	1.20	0
3	23	89	11	0	6.14	0.76	0
Average					5.39	1.01	0
Deviation					0.66	0.23	0
E.P.M					0.47	0.16	0

The results of tables 1-3 show that in none of the tested conditions a statistically significant alteration of the cell counts in pleural lavage occurred, indicating that the administration of pollen grains under these conditions does not induce the characteristic inflammatory response of allergies. In view of these results, a new battery of experiments was conducted by using a larger number of animals and a higher exposure to the pollen grains.

# 11

## EXAMPLE 4

### Immune Response of Rats Submitted to Consecutive Intrapleural Injections

TABLE 4

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Saline solution intrapleural injection (as control, 100 $\mu$ L per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in pleural lavage 4 hours after the procedure by intrapleural injection of 5 $\mu$ g of pollen grains in a volume of 100 $\mu$ L per animal.							
N	CAM Leu tot	Mono %	Neut Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	44	78	3	19	11.67	0.45	2.84
2	30	78	17	5	7.02	1.53	0.45
3	24	77	16	7	5.54	1.15	0.50
Average					8.08	1.04	1.27
Deviation					3.20	0.55	1.37
E.P.M					2.26	0.39	0.97

TABLE 5

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intrapleural injection of 5 $\mu$ g of wild tobacco pollen grains (in saline solution injection of 100 $\mu$ L per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in pleural lavage 4 hours after the procedure by intrapleural injection of 5 $\mu$ g of pollen grains in a volume of 100 $\mu$ L per animal.							
N	CAM Leu tot	Mono %	Neut Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	27	85	13	2	6.89	1.05	0.16
2	45	77	8	15	10.40	1.08	2.03
3	52	64	11	25	11.32	1.94	4.42
4	85	69	8	23	19.94	2.31	6.65
Average					12.13	1.60	3.31
Deviation					5.54	0.63	2.82
E.P.M					3.20	0.36	1.63

TABLE 6

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intrapleural injection of 50 $\mu$ g of wild tobacco pollen grains (in saline solution injection of 100 $\mu$ L per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in pleural lavage 4 hours after the procedure by intrapleural injection of 50 $\mu$ g of pollen grains in a volume of 100 $\mu$ L per animal.							
N	CAM Leu tot	Mono %	Neut Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	50	68	14	18	10.20	2.10	2.70
2	37	69	14	17	7.66	1.55	1.89
3	145	71	16	13	37.06	8.35	6.79
4	36	81	10	9	8.75	1.08	0.97
Average					15.92	3.27	3.09
Deviation					14.13	3.41	2.57
E.P.M					8.16	1.97	1.48

# 12

TABLE 7

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intrapleural injection of 500 $\mu$ g of wild tobacco pollen grains (in saline solution injection of 100 $\mu$ L per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in pleural lavage 4 hours after the procedure by intrapleural injection of 500 $\mu$ g of pollen grains in a volume of 100 $\mu$ L per animal.							
N	CAM Leu tot	Mono %	Neut Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	67	77	7	16	19.60	1.78	4.07
2	86	55	7	38	16.08	2.05	11.11
3	136	56	17	27	30.46	9.25	14.69
4	147	43	4	53	25.28	2.35	31.16
Average					22.86	3.86	15.26
Deviation					6.33	3.60	11.48
E.P.M.					3.66	2.08	6.63

The results of tables 4-7 show that there was no statistically significant alteration of the cell counts in pleural lavage under the conditions of consecutive administrations of 5 and 50  $\mu$ g of pollen grains. In the condition of administering 500  $\mu$ g of pollen grains there was a significant increase in the counts of mononuclear cells and neutrophils, as well as an increase (even though statistically not significant) of eosinophils. These results are also shown in FIG. 3, panels A, B and C, respectively. The observed increase of the cell counts in the animals submitted to the administration of 500  $\mu$ g of pollen grains demonstrates that the animals are responsive only to extreme concentrations of said pollen grains. However, the intrapleural administration of pollen grains in the concentration range of up to 50  $\mu$ g per animal, in 3 consecutive applications did not induce detectable inflammatory-type response which is typical of allergies. Since the typical conditions of intrapleural administration of antigens very rarely exceed a concentration of 20  $\mu$ g per animal, these results show that intrapleural pollen grain injections could be, surprisingly, employed in immunization or immunotherapy programs for animals, without hereby inducing the appearance of typical allergic/inflammatory symptoms. In order to verify the response of animals submitted to intranasal administration of pollen grains, another set of experiments was performed, as shown in the tables below.

## EXAMPLE 5

### Immune Response of Rats Submitted to Two Consecutive Intranasal Instillations

TABLE 8

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intranasal instillation of saline solution (as a control, 50 $\mu$ L per nostril per animal) in two consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage of normal animals, 4 hours after the last instillation.							
N	CAM Leu tot	Mono cont.	Eos cont.	Neut cont.	Mono thousands	Eos Thous.	Neut Thous.
1	74	73	9	18	1351	167	333
2	28	89	0.5	10.5	623	4	74
3	45	99	1	0	1114	11	0
4	26	30	0	70	195	0	455
Average					820.6	45.3	215.4
Deviation					515.5	80.9	214.3
E.P.M.					297.6	46.7	123.7

## 13

TABLE 9

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intranasal instillation of 5 µg of wild tobacco pollen grains (in saline solution, instillation of 50 µL per nostril per animal) in two consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	127.5	54	23	23	1721	701	765
2	64	70	0	30	1120	0	480
3	81	71	0	29	1438	0	587
4	41	97	0	3	994	0	31
5	38.5	85	0	15	818	0	144
Average					1218.3	140.3	401.5
Deviation					360.9	313.6	306.7
E.P.M.					180.5	156.8	153.4

TABLE 10

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intranasal instillation of 50 µg of wild tobacco pollen grains (in saline solution, instillation of 50 µL per nostril per animal) in two consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	223	46	0	54	2565	0	3011
2	147	62	1	38	2279	37	1397
3	37	95	0	5	879	0	46
4	30	97	0	3	728	0	23
5	68	100	0	0	1700	0	0
Average					1629.9	7.4	895.2
Deviation					943.7	18.4	1323.8
E.P.M.					409.1	8.2	661.9

TABLE 11

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intranasal instillation of 500 µg of wild tobacco pollen grains (in saline solution, instillation of 50 µL per nostril per animal) in two consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	68.5	70	0	30	1199	0	514
2	48	77	0	23	924	0	276
3	86	62	0	38	1333	0	817
4	135	62.5	0	37.5	2109	0	1266
Average					1391.3	0	718.1
Deviation					508.1	0	426.9
E.P.M.					293.3	0	246.5

## 14

EXAMPLE 6

## Immune Response of Rats Submitted to Three Consecutive Intranasal Instillations

TABLE 12

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intranasal instillation of saline solution (as a control, 50 µg per nostril, per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage of normal animals.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	49	83	0	17	1017	0	208
2	35	96	0	4	840	0	35
3	69	59	0	41	1018	0	707
4	47	84	0	6	987	0	71
5	58	57	0	43	827	0	624
Average					937.6	0	328.9
Deviation					96.2	0	315.3
E.P.M.					48.1	0	157.6

TABLE 13

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intranasal instillation of 5 µg of wild tobacco pollen grains (in saline solution, instillation of 50 µL per nostril, per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.							
N	CAM Leu tot	Mono cont.	Eos cont.	Neut cont.	Mono Thous.	Eos Thous.	Neut Thous.
1	79	53	0	47	1047	0	928
2	60	60	4	36	900	60	540
3	53.6	31	0	69	415	0	925
4	103	62	0	38	1597	0	979
Average					989.7	15	842.8
Deviation					486.3	30	203.4
E.P.M.					280.7	17.3	117.4

TABLE 14

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intranasal instillation of 50 µg of wild tobacco pollen grains (in saline solution, instillation of 50 µL per nostril, per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	119	37	0	63	1101	0	1874
2	93	88	0	12	2046	0	279
3	66	97	0	3	1601	0	50
4	86	77	3	20	1656	65	430
Average					1600.7	16.1	658.2
Deviation					387.8	32.3	825.7
E.P.M.					223.9	18.6	476.7



15

TABLE 15

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Intranasal instillation of 500 µg of wild tobacco pollen grains (in saline solution, instillation of 50 µL per nostril, per animal) in three consecutive exposures with an interval of one week between each exposure. The chart shows the cell counts in broncho-alveolar lavage 4 hours after the last intranasal instillation.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Thous.	Eos Thous.	Neut Thous.
1	83	50	0	50	1038	0	1038
2	90	82	0	18	1845	0	405
3	104	98	0	2	2548	0	52
4	53	47	0	53	623	0	702
5	45	66	0	34	743	0	383
Average					1359.2	0	515.9
Deviation					817.8	0	371.5
E.P.M.					408.9	0	185.8

The results of tables 12-15 show that in none of the tested conditions a statistically significant alteration of the cell counts in broncho-alveolar lavage occurred, indicating that the administration of pollen grains under these conditions does not induce detectable allergic-type inflammatory response. In view of these results, a new set of experiments was conducted involving a larger number of animals and higher concentrations of pollen grains. These results are also presented in FIG. 3, panels A, B and C, respectively, which clearly show that there was no statistically significant alteration of the cell counts in pleural lavage under the consecutive administration conditions of 5, 50 and 500 µg of pollen grains.

## EXAMPLE 7

Immune Response of Rats Subjected to Subcutaneous Injection, Followed by a “Booster”—Challenge with 10 µg of Pollen Grains in the Pleural Cavity

TABLE 16

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. The chart shows the cell counts in pleural lavage of normal animals, which were not submitted to any program of sensitizing or challenge.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	39	87	12	1	11.5	1.6	0.1
2	34	81	19	0	8.3	1.9	0
3	26	89	11	0	7.4	0.9	0
4	33	77	23	0	7.6	2.3	0
5	40	86	13	1	11.7	1.8	0.1
Average					9.3	1.70	0.05
Deviation					2.13	0.51	0.07
E.P.M.					1.07	0.25	0.04

16

TABLE 17

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Saline solution subcutaneous injection (according control, 100 µL per animal), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the booster, that is, on the 14 <sup>th</sup> day) through intrapleural injection of 10 µg of pollen grains in a volume of 100 µL of saline solution per animal.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	29	67	33	0	6.6	3.3	0
2	53	24	5	71	3.9	0.8	11.7
3	39	63	36	1	7.4	4.2	0.1
Average					5.97	2.76	3.93
Deviation					1.8	1.75	6.70
E.P.M.					1.27	1.24	4.74

TABLE 18

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Subcutaneous injection of 50 µg of wild tobacco - WT - pollen grains (100 µL per animal, in saline solution), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the booster, that is, on the 14 <sup>th</sup> day) through intrapleural injection of 10 µg of pollen grains in a volume of 100 µL of saline solution per animal.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	31	51	35.5	13.5	6	4.2	1.6
2	24	80	17.5	2.5	6.1	1.3	0.2
3	29	63	30.5	6.5	5.5	2.7	0.6
4	26	50	46	4	3.9	3.3	0.6
5	28	82	14	4	7.3	1.3	0.4
Average					5.78	2.54	0.67
Deviation					1.25	1.26	0.54
E.P.M.					0.63	0.63	0.27

TABLE 19

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Subcutaneous injection of 500 µg of wild tobacco - WT - pollen grains (100 µL per animal, in saline solution), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the booster, that is, on the 14 <sup>th</sup> day) through intrapleural injection of 10 µg of pollen grains in a volume of 100 µL of saline solution per animal.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	48	49	6	45	8	0.98	7.34
2	47	53	10	37	8.97	1.69	6.26
3	61	76	5	19	14.8	0.98	3.71
4	33	89	10	1	8.8	0.99	0.10
5	41	62	3	35	8.6	0.42	4.88
Average					9.85	1.01	4.46
Deviation					2.81	0.45	2.80
E.P.M.					1.41	0.23	1.40

17

TABLE 20

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Subcutaneous injection of 50 µg of wild tobacco - WT - pollen grains (100 µL per animal, in saline solution with 5 mg of aluminum hydroxide as adjuvant), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the booster, that is, on the 14 <sup>th</sup> day) through intrapleural injection of 10 µg of pollen grains in a volume of 100 µL of saline solution per animal.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	49	77	8	15	13.58	1.41	2.65
2	23	76	17	7	5.94	1.33	0.55
3	49	73	13	14	11.09	1.97	2.13
4	33	82	15	3	8.12	1.49	0.30
5	56	56	15	29	9.41	2.52	4.87
Average					9.63	1.74	2.10
Deviation					2.90	0.50	1.85
E.P.M.					1.45	0.25	0.92

TABLE 21

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Subcutaneous injection of 500 µg of wild tobacco - WT - pollen grains (100 µL per animal, in saline solution with 5 mg of aluminum hydroxide as adjuvant), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the booster, that is, on the 14 <sup>th</sup> day) through intrapleural injection of 10 µg of pollen grains in a volume of 100 µL of saline solution per animal.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	47	67	2	31	11.34	0.34	5.25
2	94	62	5	33	22.15	1.79	11.79
3	32	68	3	29	6.96	0.31	3
4	56	69	4	27	12.36	0.72	4.84
5	52	71	18	11	11.08	2.81	1.72
Average					12.78	1.19	5.31
Deviation					5.63	1.08	3.89
E.P.M.					2.81	0.54	1.95

## EXAMPLE 8

Immune Response of Rats Subjected to Subcutaneous Injection, Followed by a “Booster”—Challenge with 20 µg of Pollen Grains in the Pleural Cavity

TABLE 22

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Subcutaneous injection of saline solution (according to control 100 µL per animal), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage of normal animals, which were not subjected to any program of sensitizing or challenge.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	15	89	11	0	4.3	0.5	0
2	25	84	16	0	6.3	1.2	0
3	14	71	25	4	3.2	1.1	0.2
4	21	88	12	0	5.9	0.8	0
Average					4.92	0.91	0.04
Deviation					1.45	0.31	0.09
E.P.M.					0.84	0.18	0.05

18

TABLE 23

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Subcutaneous injection of saline solution (according to control 100 µL per animal), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure (7 days after the booster, that is, on the 14 <sup>th</sup> day) through intrapleural injection of 20 µg of pollen in a volume of 100 µL of saline solution per animal.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	34	69	31	0	7.0	3.2	0
2	21	81	18	1	5.3	1.3	0.1
3	48	87	11	2	13.2	1.8	0.3
4	28	65	31	4	5.8	2.8	0.4
5	12	78	20	2	3	0.8	0.1
Average					6.88	1.98	0.16
Deviation					3.83	0.99	0.16
E.P.M.					1.92	0.50	0.08

TABLE 24

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Subcutaneous injection of 50 µg of wild tobacco pollen grains (100 µL per animal, in a saline solution with 5 mg of aluminum hydroxide as adjuvant), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure through intrapleural injection of 20 µg of pollen grains in a volume of 100 µL of saline solution per animal.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	22	78	21	1	5.5	1.5	0.1
2	40	71.5	26.5	2	8.6	3.2	0.2
3	16	78	21	1	4.2	1.1	0.1
4	19	88	12	0	5.7	0.8	0
5	19	81	10	9	5.2	0.6	0.6
6	24	75	17	8	5.9	1.3	0.6
Average					12.96	1.43	0.26
Deviation					6.17	0.92	0.28
E.P.M.					2.76	0.41	0.12

TABLE 25

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Subcutaneous injection of 100 µg of wild tobacco pollen grains (100 µL per animal, in saline solution with 5 mg of aluminum hydroxide as adjuvant), followed by a booster in the same conditions after 7 days. The chart shows the cell counts in pleural lavage 4 hours after the procedure through intrapleural injection of 20 µg of pollen grains in a volume of 100 µL of saline solution per animal.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	32	81	16	3	8.81	1.74	0.33
2	15	80.5	19	0.5	4.11	0.97	0.03
3	19	84	15	1	5.11	0.91	0.06
4	27	69	27	4	5.96	2.33	0.35
5	23.25	78	22	0	6.89	1.9	0
6	23.25	80	9	11	6.32	0.7	0.87
Average					6.20	1.43	0.27
Deviation					1.61	0.66	0.33
E.P.M.					0.72	0.29	0.15

## EXAMPLE 9

Immune Response of Rats Subjected to Subcutaneous Injection of Pollen Grains, Followed by a Booster—Challenge Through Subplantar Injection of 20 µg of Pollen Grains

## 19

Two groups of animals were subjected to a subcutaneous injection (100  $\mu$ L per animal) of pollen grain suspensions in the following conditions:

1—Subcutaneous injection of 50  $\mu$ g of pollen grains in a saline solution and aluminum hydroxide as adjuvant (5 mg per animal); and 2—Subcutaneous injection of 100  $\mu$ g of pollen grains in a saline solution and aluminum hydroxide as adjuvant (5 mg per animal).

One week after these subcutaneous injections (day 7) the groups of animals were subjected to a new subcutaneous injection (booster) in the same respective pollen grain concentrations, but without an adjuvant. After one week (day 14), all animal groups were challenged by sub plantar injection (50  $\mu$ L) of 20  $\mu$ g suspensions of pollen grains. There was no appearance of edemas on the sole of the paws/feet in any of the evaluated conditions.

## EXAMPLE 10

Immune Response of Rats Subjected to Intranasal Instillation of Pollen Grains, Followed by a Booster—Challenge Through Subplantar Injection of 20  $\mu$ g of Pollen Grains

Four groups of animals were subjected to an intranasal instillation (50  $\mu$ L per nostril) of pollen grain suspensions under the following conditions:

1—Intranasal instillation of 50  $\mu$ g of pollen grains in saline solution;

2—Intranasal instillation of 25  $\mu$ g of pollen grains in saline solution;

3—Intranasal instillation of 50  $\mu$ g of pollen grains in saline solution and aluminum hydroxide as adjuvant (5 mg per animal); and

4—Intranasal instillation of 25  $\mu$ g of pollen grains in saline solution and aluminum hydroxide as adjuvant (5 mg per animal).

One week after these nasal instillations (day 7) the animal groups were subjected to another intranasal administration (booster) with the same respective concentrations of pollen grains, but without adjuvants. After one week (day 14), all animal groups were challenged by a sub plantar injection (50  $\mu$ L) of suspensions of 20  $\mu$ g of pollen grains. There was no occurrence of paw/feet edemas in any of the evaluated conditions.

## EXAMPLE 11

Immune Response of Rats Subjected to Consecutive Intranasal Instillations with WT and GM Tobacco Pollen Grains

Groups of animals were subjected to intranasal instillations (50  $\mu$ L per nostril) of wild pollen (WT) suspensions or transgenic pollen suspensions (GM—whose pollen grains contain the GUS protein) in the concentrations of 0, 5 and 50 total pollen  $\mu$ g per animal. Three instillations were performed with intervals of 7 days, after which a broncho-alveolar washing as well as a cellular counting was performed

TABLE 26

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Nasal instillation of a saline solution (control of 50 $\mu$ L per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	7	70	0	30	0.123	0	0.053
2	6	96	0	4	0.144	0	0.006
3	10	60	0	40	0.15	0	0.1

## 20

TABLE 26-continued

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Nasal instillation of a saline solution (control of 50 $\mu$ L per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
Average					0.138	0	0.053
Deviation					0.014	0	0.047
E.P.M.					0.01	0	0.033

TABLE 27

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Nasal instillation of 5 $\mu$ g of wild tobacco pollen grains - WT - (in a saline solution, instillation of 50 $\mu$ L per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	54	51	0	49	0.689	0	0.662
2	50	48	0	52	0.6	0	0.65
3	27	55	0	45	0.371	0	0.304
4	28	43	0	57	0.301	0	0.399
Average					0.49	0	0.503
Deviation					0.184	0	0.18
E.P.M.					0.106	0	0.104

TABLE 28

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Nasal instillation of 5 $\mu$ g of transgenic tobacco pollen grains - GM - (in a saline solution, instillation of 50 $\mu$ L per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	48	30	0	70	0.36	0	0.84
2	29	58	0	42	0.421	0	0.305
3	75	37	1	62	0.694	0.019	1.163
4	39	63	0	37	0.614	0	0.361
Average					0.522	0.005	0.667
Deviation					0.157	0.009	0.408
E.P.M.					0.091	0.005	0.236

TABLE 29

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Nasal instillation of 50 $\mu$ g of wild tobacco pollen grains - WT - (in a saline solution, instillation of 50 $\mu$ L per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	172	4	0	96	0.172	0	4.128
2	24	51	0	49	0.306	0	0.294
3	109	20	0	80	0.545	0	2.180

21

TABLE 29-continued

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Nasal instillation of 50 µg of wild tobacco pollen grains - WT - (in a saline solution, instillation of 50 µL per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
4	39	66	0	34	0.644	0	0.332
5	104	56	0	44	1.456	0	1.144
Average					0.624	0	1.615
Deviation					0.216	0	1.6
E.P.M.					0.25	0	0.8

TABLE 30

Experimental conditions of pollen grain exposure to <i>R. novergicus</i> Wistar rats and their respective results. Nasal instillation of 50 µg of transgenic tobacco pollen grains - GM - (in a saline solution, instillation of 50 µL per nostril, per animal) in three consecutive exposures with intervals of one week between each exposure. The chart presents the cell counts in lavage broncho-alveolar 4 hours after the last instillation under the above-described conditions.							
N	CAM Leu tot	Mono %	Eos %	Neut %	Mono Millions	Eos Millions	Neut Millions
1	31	92	0	8	0.713	0	0.062
2	65	70	0	30	1.138	0	0.488
3	22	44	3	53	0.242	0.02	0.292
4	40	48	0	52	0.480	0	0.52
5	35	55	0	45	0.481	0	0.394
Average					0.610	0.003	0.351
Deviation					0.338	0.007	0.184
E.P.M.					0.169	0.004	0.092

The results of tables 26-30 are better visualized in FIG. 3, panels D-F, and indicate that in none of the pollen grain concentrations administered by intranasal means any significant alteration in the cell counts (which would be typical of allergic-inflammatory reactions) occurred. The statistically significant difference among the mononuclear cells and neutrophil counts after the administration of pollen grains WT and GM (FIG. 3, panels D and E) is surprising and favorable in regard to the present invention's purposes, since it demonstrates that the pollen grains with modified composition (GM), when used in concentrations of 50 µg, would have less potential to induce inflammation in the broncho-alveolar cavity than wild tobacco pollen grains (WT).

## EXAMPLE 12

Immune Response of Rats Subjected to Different Programs of Pollen Grain Administration—Measurement of the Total Seric IgG Through ELISA

Table 31 shows the seric IgG concentration data of the animals subjected to the conditions described in Example 6, tables 12-15.

22

TABLE 31

Concentration data of the total seric IgG, in µg/mL, in serum samples of animals subjected to three consecutive intranasal administrations, with an interval of one week between each application of wild type tobacco pollen grain (WT) suspensions in different concentrations.				
Animal	Saline solution	WT 5 µg	WT 50 µg	WT 500 µg
1	1125.6	1441.5	1123.3	1012.7
2	1367	1337.7	1459.5	1461.8
3	1376	1084.9	1414.4	1152.6
4	1545.3	836.7	1511.4	918
5	1504.6			1015
6				1488.9
N	5	4	4	6
Average	1383.7	1175.2	1377.2	1174.8
Deviation	164.1	270.8	173.8	244.7
E.P.M.	82.0	156.4	100.4	109.4

Table 32 shows the seric IgG concentration data of the animals subjected to the conditions described in Example 7, tables 16 and 19-21.

TABLE 32

Concentration data of the total seric IgG, in µg/mL, in serum samples of animals subjected to an intrapleural injection with different concentrations of wild type tobacco pollen grains (WT), with or without adjuvant (AlOH <sub>3</sub> ), followed by a booster one week later, under the same conditions and challenge, and, one week thereafter by an intrapleural injection of 10 µg of pollen grains.				
Animal	Saline solution	500 µg	50 µg + AlOH <sub>3</sub>	500 µg + AlOH <sub>3</sub>
1	942.8	956.3	981.1	951.8
2	807.4	665.2	906.7	1037.5
3	1024	836.7	678.8	1367
4	784.8	884.1	1100.7	960.8
5			954.1	1673.9
N	4	4	5	5
Average	889.8	835.6	924.3	1198.2
Deviation	113.5	123.8	154.8	315.3
E.P.M.	65.5	71.5	77.4	157.7

Table 33 shows the seric IgG concentration data of the animals subjected to the conditions described in Example 11, tables 26-30.

TABLE 33

Concentration data of the total seric IgG, in µg/mL, in serum samples of animals subjected to 3 (three) consecutive intranasal administrations, with an interval of one week between each application of wild type tobacco pollen grain (WT) suspensions or genetically modified (GM) in different concentrations.					
Animal	Saline solution	WT 5 µg	WT 50 µg	GM 5 µg	GM 50 µg
1		1098.5	1069.1	967.6	523.1
2	1130.1	1457.3	1247.4	1062.4	784.8
3	1470.8	1078.2	931.5	1299.3	978.9
4	1283.5	1367	958.6	1048.8	893.1
5	1479.8	723.9	1103		744.2
N	4	5	5	4	5
Average	1341.1	1145.0	1061.9	1094.5	784.8
Deviation	167.2	287.6	126.3	142.8	172.9
E.P.M.	96.6	143.8	63.2	82.4	86.4

## 23

## EXAMPLE 13

Analysis of the Immune Response of Rats Subjected to Consecutive Subcutaneous Injections of Pollen Grains, Recognized as Allergens (*Betula fontinalis*)

Aiming to subject the animals to conditions in which an inflammatory and/or allergic response would be more likely, so as to eliminate the hypothesis that the animals utilized in the tests would not be responsive, new trials were conceived with the use of pollen grains reported in literature to be associated with allergic events (Ahlholm et al, "Genetic and Environmental Factors Affecting the Allergenicity of Birch (*Betula pubescens* ssp. *czerepanovii* [Orl.] Hamet-ahiti) Pollen" Clin. Exp. Allergy 28:1384-1388, 1998). In this sense, the pollen grains of *Betula fontinalis* were commercially acquired and utilized in experiments with rats as follows:

Groups of 5 (five) animals were subjected to the exposure of pollen grains in the following conditions:

Group 1—Subcutaneous Injection (100  $\mu$ L Per Animal):

*Betula* pollen suspension (100  $\mu$ g/animal) in saline solution;

*Betula* pollen suspension (100  $\mu$ g/animal) in saline solution+Al(OH)<sub>3</sub> (5 mg/animal); and

Wild tobacco pollen suspension—WT (100  $\mu$ g/animal) in saline solution+Al(OH)<sub>3</sub> (5 mg/animal).

Group 2—Instillation (50  $\mu$ L Per Nostril):

*Betula* pollen suspension (100  $\mu$ g/animal) in saline solution+Al(OH)<sub>3</sub> (5 mg/animal).

After eight (8) weeks of consecutive exposures under the above-described conditions, Group 1 was subjected to a challenge, by injection into the footpad, of 50  $\mu$ g of pollen grain suspension (50  $\mu$ L) and measurement of the animal's feet volume in a plethysmograph. The results of Table 34 indicate the occurrence of edemas in the animals subjected to subcutaneous injections of WT tobacco pollen and, to a lesser degree, with *Betula* pollen.

TABLE 34

The volumes, in ml, of the right (control) and the left paws/feet (challenge) were measured in a plethysmograph. The rats were subjected to 8 (eight) consecutive subcutaneous injections with pollen grains under the above-described conditions, followed by a challenge through a sub plantar injection of 50 $\mu$ g of pollen grain suspension (50 $\mu$ L per paw). The presented averages represent the volume differences between the left and right paws.					
Tobacco WT + Al(OH) <sub>3</sub>		<i>Betula</i> in saline solution		<i>Betula</i> + Al(OH) <sub>3</sub>	
Right	Left	Right	Left	Right	Left
1.23	1.79	1.29	1.54	1.24	1.36
1.23	1.80	1.35	1.58	1.38	1.28
1.30	1.75	1.28	1.48	1.31	1.34
1.20	1.30			1.40	1.42
				1.32	1.29
Average	0.420		0.227		0.008
Deviation	0.220		0.025		0.081
E.P.M.					

After eight consecutive weeks of nasal instillations in the animals of Group 2, broncho-alveolar lavages were used to count the cells under the above-described conditions. The cell counts indicated that there was no change in relation to the control animals. Taken together these results point to the following conclusions: (i) the allergic response to subcutaneously injected pollen grains only occurs after an elevated number of consecutive exposures and tends to be higher in the absence of an adjuvant; (ii) it was not possible to detect typical alterations of allergic responses in any of the nasal

## 24

instillation conditions in rats. Since edemas indeed occurred under the subcutaneous injection condition (with WT tobacco pollen and also with *Betula* pollen) and the absence of typical allergic alterations through instillation in all of the conditions evaluated in the present invention, the results indicate that there seems to be a relation between the antigen presentation route and the pattern of response.

## EXAMPLE 14

Immune Response of Rats Subjected to Different Programs of Pollen Grains Administration—Detection of Specific Seric IgG Against of Pollen Grain Proteins Through Western Blot

The formation of specific seric IgG against pollen grain proteins was monitored through Western Blot, in which total extracts of pollen WT (wild) and GM (containing the GUS enzyme) proteins were separated through electrophoresis, followed by the transference of the bands to a nitrocellulose membrane. The serum of the animals subjected to three consecutive instillations, in intervals of one week each, with pollen WT or GM, in pool, were then incubated separately, with said membrane (previously cut into pieces to allow the separation of the samples), which, after being washed, was incubated in the presence of secondary anti-rat IgG antibodies marked with alkaline phosphatase. The colorimetric reaction with adequate substrates indicated the detection of specific IgG's against pollen grain proteins. As shown on Table 35, the serum of the animals instilled with tobacco pollen grains "WT" and also in the serum of the animals instilled with tobacco pollen grains "GM" revealed the presence of IgG type antibodies specific against proteins of said pollen grains. Taken together, the results of these experiments point to the feasibility of the use of pollen grains as modulators of the immune response in mammals. More specifically, the results point to the feasibility of the use of whole pollen grains as stimulators of protective response (formation of specific seric IgG against pollen grain proteins) in mammals.

TABLE 35

Detection, through Western Blot, of specific seric IgG against proteins from tobacco pollen grains WT and GM.				
	Condition of exposure			
	Saline solution	Pollen WT 5 $\mu$ g	Pollen GM 5 $\mu$ g	Pollen GM 50 $\mu$ g
seric IgG	—	+	+	—

The approach using pollen grains has, besides other advantages mentioned in the present invention, the important characteristic of requiring very small amounts of vegetal material for immunization. The results of Table 35 show the formation of specific seric IgG against proteins present in pollen grains after three (3) administrations of 5  $\mu$ g of pollen grains. Even though no study was so far performed in order to define the minimum amounts necessary to induce the immune response observed on Table 35, the present results offer a reasonable base to estimate the amount of pollen grains that would be necessary for the immunization in other species, such as humans. It is known that the human immune system is considered to be from 100 to 1.000 times more sensitive than that of murines, which means that, by maintaining the proportion between the administered amount and the body weight, just some dozens or hundreds of square meters of cultivation would be sufficient to produce vaccines for entire popula-

tions. This allows, among other things, the adoption of simple containment measures in order to decrease biosafety concerns. Further, it is convenient to remember that several other strategies for pollen grain containment are available, such as irradiation, the use of suicidal genes, and infertility barriers. The present invention's strategy is particularly applicable for animal vaccinations, being also useful to fight epidemics and prevent or remedy situations which might result from the use of biological weapons or terrorist attacks.

#### EXAMPLE 15

##### Obtaining Genetic Constructions for the Transformation of Plants Destined to Produce Pollen Grains with Heterologous Polypeptides on their External Surface

In another preferred embodiment of the present invention, the presence of heterologous polypeptides on the external surface of pollen grains is desirable because it is related to the use of pollen grains in in vitro antigen-antibody type reactions, that is, reactions used for in vitro immune diagnosis. In order to evaluate this applicability, *A. thaliana* plants were transformed with gene constructions which would direct the expression of heterologous polypeptide in a subcellular localization compatible with its presence on the external surface of the pollen grains.

In order to fulfill these requirements, in one of the preferred embodiments of the present invention, the coding gene of the heterologous polypeptide promoter SEQ ID NO. 2) in question is translationally fused to the coding sequence of the AtGRP17 gene (SEQ ID NO. 1), while said fusion is controlled by at least part of the promoter region (SEQ ID NO. 3) of the AtGRP17, this promoter being able to direct the gene fusion expression in the anther's tapetum. In order to prepare said gene construction, the following steps were taken:

For the amplification of the promoter region of the AtGRP17 and its ORF specific oligonucleotides were used: RR1f (5'ATA AAG CTT TTT CTC TGT TTT TGT CCG TGG AAC) (SEQ. ID NO. 4) and RR2r (5'ATA CCA TGG CAC GTG ATT CGG TGG AAG TCC TGC C) (SEQ. ID NO. 5). The plasmid pC027 (described by Olivera et al "Inflorescence-specific genes from *Arabidopsis thaliana* encoding glycine-rich proteins". Plant J. 3: 495-507, 1993; Franco et al., "Distal regulatory regions restrict the expression of cis-linked gene to the tapetal cells". FEBS Letters 25965: 1-6, 2002) was used as target for the amplification, by PCR, of the promoter region and of the AtGRP17 ORF. By using the oligonucleotides RR1f and RR2r the product of amplification ProAtGRP67 (FIG. 6) was obtained and, after cleavage with the enzymes XbaI and NcoI, was linked to plasmid pCambia cleaved with the same enzymes (FIG. 5), thus originating the construction pProAtGRP17\_GUSGFP (FIG. 7).

#### EXAMPLE 16

##### Transformation of *A. thaliana* and Analysis of the Heterologous Polypeptide's Localization

The gene construction of the previous example was introduced in *E. coli* and in *Agrobacterium tumefaciens* so as to later transform *A. thaliana*. Plant transformation confirmation was performed through the extraction of genomic DNA by methodologies known to skilled in the art and by PCR-mediated amplification of the heterologous regions introduced into the transformed plant. Plant transformation was also confirmed by the expression analysis of GUS enzyme activity in the intended subcellular localizations, by means of a calorimetric reaction using X-Gluc as substrate and the

corresponding blue color formation. Plant tissue samples were observed by stereoscopic and optical microscopy.

The gene construction (FIG. 7) containing the reporter genes gus and gfp, was introduced in *E. coli* by electroporation and in *A. tumefaciens* by thermal shock. The cloning was confirmed by the analysis of the expected profile of fragments generated by the digestion with restriction enzymes (FIG. 8). *A. thaliana* inflorescences were inoculated with cultures of *A. tumefaciens* containing said gene construction (transformation technique called "floral dip", described by Clough & Bent "Floral dip: a Simplified Method for *Agrobacterium*-mediated Transformation of *Arabidopsis thaliana*" The Plant Journal 16(6):735-743, 1998). The seeds generated after that procedure were collected and sown on plaques containing a selective medium comprising hygromycin. The plantlets selected from the plaques were transplanted into the soil in phytotron, where they were cultivated in conditions which favor self-fertilization. Analysis of these plants' inflorescences by microscopy (FIG. 9 A-D) confirmed the presence of GUS activity in the expected subcellular localization (by means of the corresponding calorimetric reaction described in Example 2). More specifically, the activity of the GUS enzyme was only detected in the later stages of the anther's development (FIG. 9 A), and still more specifically on the anther's tapetum (FIG. 9 C).

#### EXAMPLE 17

##### Detection of the Heterologous Polypeptide on the Surface of Pollen Grains

The transformation of *A. thaliana* plants according to Example 16 resulted in the generation of 11 lineages effectively producing heterologous polypeptides in the pollen grains. Those lineages were cultivated in phytotron and self-pollinated so as to allow the later obtention of hundreds of seeds. Seeds of each one of the lineages were sown directly into the soil and the resulting plants were cultivated in phytotron. After inflorescence formation entire flowers were collected in Eppendorf tubes with the help of a scalpel. 100 µL of phosphate buffer 0.1 M pH 7 was added to said tubes, which were then vortexed in order to allow the formation of homogeneous pollen grain suspensions. Afterwards, 1 µL of the X Gluc reagent was added and, 30 minutes at 37° C. thereafter, the presence of the GUS enzyme triggered the formation a blue-colored complex. Fractions of these suspensions were then collected with micropipette and put on glass slides for observation under the microscope. The result of that observation (FIG. 9 D) indicated the presence of the active heterologous polypeptide (GUS enzyme) in the pollen grain. These results point collectively to the feasibility of the use of whole pollen grains as reagents for immune diagnosis.

The skilled persons will understand from the present description that any heterologous polypeptide can be produced by the processes of the present invention, including post-translationally modified polypeptides, such as glycosylated proteins and the like. Similarly, combinations of heterologous polypeptides or translational fusions of heterologous polypeptide segments may be produced by the teachings of the present invention, including, but not limiting to, at least part of polypeptides derived from eukaryotic organisms such as mammals including humans, plants, parasites, fungi or derived from procaryotic organisms such as bacteria or even viruses, as well as combinations thereof, regardless of being natural or synthetic polypeptides. Therapeutic peptides preferred for the purposes of the present invention include pep-

tidic hormones, cytokines, interleucins, antibodies (and/or fragments thereof) and combinations thereof. The heterologous polypeptides produced by these processes may be used in several immunoreactions, including the immunomodulation of mammals and in vitro immunodiagnostic reactions.

The referred immunomodulation may have an immunotherapeutic, immunoprotective or vaccination purpose, according to the chosen immunogen and/or according the combination of the chosen immunogens.

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 SEQUENCE LISTING
 

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<210> SEQ ID NO 1

<211> LENGTH: 1818

<212> TYPE: DNA

<213> ORGANISM: *Arabidopsis thaliana*

<400> SEQUENCE: 1

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tctctttcag agagagatgg aagaaagttt tcttttctca gtatgttctc ttttctcatg	180
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tgcagacgta ggtggggagt aaagccgaag gataatccac ctccggcagg acttccaccg	660
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atgtccggaa gtggaggagg catgtcagga agtgaaggag gtgtgtcttg aagtgaagga	1560
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aaacacggag gtcttagagg taaattcggg aagaaaagag gtatgtcagg aagtgaagga	1680
ggtatgtctg gaagtgaagg aggtatgtcg gaaagtggta tgtccgggag tggaggggggt	1740
aaacacaaaa tcggaggagg taaacacaaa tttggaggag gtaaacacgg aggtggagggt	1800
ggccacatgg cgagataa	1818

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<210> SEQ ID NO 2  
 <211> LENGTH: 1569  
 <212> TYPE: DNA  
 <213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 2

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acaaagaaat taactatgaa acaatgcttt gtttaaatga agtaattaat cgggtactata    60
gcgtatatac atagaatgga tccaatttaa ccaaagcaac tgtatgtgac tatgtgaatg    120
attcaatcgt gagacattga aattgtcgtt tctccattac ctttttggaa gaaaaaccat    180
cgaaagctag ctaagacttt ttttattaaa cgaacttgct actatttcta tgttttcttt    240
gaaatgaaaa ttaaatgtgt tactgtttca ctaaaaactc aaaagtattg ctttttaatt    300
ttattattaa gaaaaactaa tcttatttat gttaagaaac ctgtcaattt ttcattgtta    360
atttcggctc tataattatt aattaacaat caatttctca aaaattgcaa tcatgattat    420
gattagatat atattagttg gattgtgatg cattttttgt aatataaaat ggatgtttgt    480
attagtttct cactcatgta attaaacacc aaatgctaga aactagtact tttgtttctc    540
agctctcgtc tattgttata tctgcaacac gaacaaaaac cttatctagg tgttatatat    600
cacggttatg tttatgagtt agaagggatt cttcaacaaa aatcacggaa ctactgtat    660
atatgtatgt gtgtatccga tcgaggttga cttccggggt tggacgttga agaagacgaa    720
ttcattgatt gggccttatat atgggcatgt attacttggt tcaagtttgt aacactttta    780
gctttttcaa ttctattoga aaccaaataa ttgggctata tatctttata caaccttcaa    840
gataaattgg accaatttta gaagagcaaa ttgaaccggg ccgttagcgt tagccaaacc    900
ccaactcctt ttcagtacaa ttaaatcaag aatttctaat aaatcgtgaa tttctagaca    960
tacatatcat aatttcgtca aagcgagcct acacctagtt ttgagctaca taactctttt   1020
cttttttttt ttatgattag gaggtttcaa aacccttgga cccataattt cttataatta   1080
gttttgtaat actaaattta ccattgagag cgacctctcg tcaactagtaa ttcgaagatc   1140
tcatattcat gacctatatt aaccatcttc cagtcaagta atttcaatcg aaattcatca   1200
aaatcatata ttttaacttag taatcacata tgatatggct aatatacgta atataacgat   1260
aaagatttct tcacgctttg atattccata aagcaatgga aatatggaat ggaagaaaac   1320
atttgaattt tacaagaaac aataaataga aggcctacaa aacatgacaa cccacacaca   1380
cacacacgaa aagagaaaat ataaagaagg acatgtaacg tgacgtagcg tagatctcca   1440
ttcactccaa tcgttttgca tggagcatgc atgtgtgtgt accgtgcacg tagtagagac   1500
cacacaactc cttcataaaa gccctctctc tcttaaccat accaaaacac aacaatccga   1560
tcagaaaaat                                     1569

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<210> SEQ ID NO 3  
 <211> LENGTH: 543  
 <212> TYPE: PRT  
 <213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 3

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Met Ser Glu Glu Leu Ser Gln Lys Pro Ser Ser Ala Gln Ser Leu Ser
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Leu Arg Glu Gly Arg Asn Arg Phe Pro Phe Leu Ser Leu Ser Gln Arg
          20          25          30

Glu Gly Arg Phe Phe Pro Ser Leu Ser Leu Ser Glu Arg Asp Gly Arg
          35          40          45

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Lys	Phe	Ser	Phe	Leu	Ser	Met	Phe	Ser	Phe	Leu	Met	Pro	Leu	Leu	Glu
50						55					60				
Val	Ile	Lys	Ile	Ile	Ile	Ala	Ser	Val	Ala	Ser	Val	Ile	Phe	Val	Gly
65					70					75					80
Phe	Ala	Cys	Val	Thr	Leu	Ala	Gly	Ser	Ala	Ala	Ala	Leu	Val	Val	Ser
			85					90						95	
Thr	Pro	Val	Phe	Ile	Ile	Phe	Ser	Pro	Val	Leu	Val	Pro	Ala	Thr	Ile
			100					105						110	
Ala	Thr	Val	Val	Leu	Ala	Thr	Gly	Phe	Thr	Ala	Gly	Gly	Ser	Phe	Gly
			115				120					125			
Ala	Thr	Ala	Leu	Gly	Leu	Ile	Met	Trp	Leu	Val	Lys	Arg	Arg	Met	Gly
			130			135					140				
Val	Lys	Pro	Lys	Asp	Asn	Pro	Pro	Pro	Ala	Gly	Leu	Pro	Pro	Asn	Ser
145					150					155					160
Gly	Ala	Gly	Ala	Gly	Gly	Ala	Gln	Ser	Leu	Ile	Lys	Lys	Ser	Lys	Ala
				165					170					175	
Lys	Ser	Lys	Gly	Gly	Leu	Lys	Ala	Trp	Cys	Lys	Lys	Met	Leu	Lys	Ser
			180					185					190		
Lys	Phe	Gly	Gly	Lys	Lys	Gly	Lys	Ser	Gly	Gly	Gly	Lys	Ser	Lys	Phe
		195					200					205			
Gly	Gly	Lys	Gly	Gly	Lys	Ser	Glu	Gly	Glu	Glu	Gly	Met	Ser	Ser	Gly
		210				215					220				
Asp	Glu	Gly	Met	Ser	Gly	Ser	Glu	Gly	Gly	Met	Ser	Gly	Gly	Glu	Gly
225					230					235					240
Gly	Lys	Ser	Lys	Ser	Gly	Lys	Gly	Lys	Leu	Lys	Ala	Lys	Leu	Glu	Lys
				245					250					255	
Lys	Lys	Gly	Met	Ser	Gly	Gly	Ser	Glu	Ser	Glu	Glu	Gly	Met	Ser	Gly
			260					265					270		
Ser	Glu	Gly	Gly	Met	Ser	Gly	Gly	Gly	Gly	Ser	Lys	Ser	Lys	Ser	Lys
		275					280					285			
Lys	Ser	Lys	Leu	Lys	Ala	Lys	Leu	Gly	Lys	Lys	Lys	Gly	Met	Ser	Gly
		290				295					300				
Gly	Met	Ser	Gly	Ser	Glu	Glu	Gly	Met	Ser	Gly	Ser	Glu	Gly	Gly	Met
305					310					315					320
Ser	Ser	Gly	Gly	Gly	Ser	Lys	Ser	Lys	Ser	Lys	Lys	Ser	Lys	Leu	Lys
				325					330					335	
Ala	Lys	Leu	Gly	Lys	Lys	Lys	Gly	Met	Ser	Gly	Gly	Met	Ser	Gly	Ser
			340					345					350		
Glu	Glu	Gly	Met	Ser	Gly	Ser	Glu	Gly	Gly	Met	Ser	Gly	Gly	Gly	Gly
		355					360					365			
Gly	Lys	Ser	Lys	Ser	Arg	Lys	Ser	Lys	Leu	Lys	Ala	Lys	Leu	Gly	Lys
		370				375					380				
Lys	Lys	Cys	Met	Ser	Gly	Gly	Met	Ser	Gly	Ser	Glu	Gly	Gly	Met	Ser
385					390					395					400
Gly	Ser	Glu	Gly	Gly	Ile	Ser	Gly	Gly	Gly	Met	Ser	Gly	Gly	Ser	Gly
				405					410					415	
Ser	Lys	His	Lys	Ile	Gly	Gly	Gly	Lys	His	Gly	Gly	Leu	Gly	Gly	Lys
			420					425					430		
Phe	Gly	Lys	Lys	Arg	Gly	Met	Ser	Gly	Ser	Gly	Gly	Gly	Met	Ser	Gly
			435				440					445			
Ser	Glu	Gly	Gly	Val	Ser	Gly	Ser	Glu	Gly	Ser	Met	Ser	Gly	Gly	Gly
					450		455				460				
Met	Ser	Gly	Gly	Ser	Gly	Ser	Lys	His	Lys	Ile	Gly	Gly	Gly	Lys	His

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465	470	475	480
Gly Gly Leu Arg Gly Lys Phe Gly Lys Lys Arg Gly Met Ser Gly Ser	485	490	495
Glu Gly Gly Met Ser Gly Ser Glu Gly Gly Met Ser Glu Ser Gly Met	500	505	510
Ser Gly Ser Gly Gly Gly Lys His Lys Ile Gly Gly Gly Lys His Lys	515	520	525
Phe Gly Gly Gly Lys His Gly Gly Gly Gly Gly His Met Ala Glu	530	535	540
<p>&lt;210&gt; SEQ ID NO 4 &lt;211&gt; LENGTH: 33 &lt;212&gt; TYPE: DNA &lt;213&gt; ORGANISM: Artificial Sequence &lt;220&gt; FEATURE: &lt;223&gt; OTHER INFORMATION: cDNA sequence (primer), sequence named as RR1f</p>			
<p>&lt;400&gt; SEQUENCE: 4</p>			
ataaagcttt ttctctgttt ttgtccgtgg aac			33
<p>&lt;210&gt; SEQ ID NO 5 &lt;211&gt; LENGTH: 34 &lt;212&gt; TYPE: DNA &lt;213&gt; ORGANISM: Artificial Sequence &lt;220&gt; FEATURE: &lt;223&gt; OTHER INFORMATION: cDNA sequence (primer), sequence named as RR2r</p>			
<p>&lt;400&gt; SEQUENCE: 5</p>			
ataccatggc acgtgattcg gtggaagtcc tgcc			34

- The invention claimed is:
1. A pharmaceutical product for vaccination of vertebrates, the pharmaceutical product comprising genetically modified pollen grains, the pollen grains comprising:

a) a promoter consisting of nucleotides 954 to 1569 of the promoter sequence SEQ ID NO. 2 operably linked to the coding sequence SEQ ID NO. 1, whereby the coding sequence SEQ ID NO. 1 is under control of the last 616 bp of the promoter sequence SEQ ID NO. 2;

b) the coding sequence SEQ ID NO. 1 being fused in-frame to a coding sequence for a heterologous polypeptide, wherein both the promoter sequence and the coding

35

sequence are present in the genome of the pollen grains; and

c) the polypeptide of sequence SEQ ID NO. 3 translationally fused to the heterologous polypeptide, the heterologous polypeptide being present in the outer surface of the pollen grain.

2. The pharmaceutical product according to claim 1, wherein the heterologous polypeptide is selected from the group consisting of eukaryotic antigens, prokaryotic antigens, viral antigens, synthetic antigens, therapeutic peptides, peptidic hormones, cytokines, interleukins, and combinations thereof.

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